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METHODS AND DEVICES FOR THE
For: ISOLATION AND ANALYSIS OF CELLULAR
PROTEIN CONTENT

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SECOND PRELIMINARY AMENDMENT

Please enter the following amendment in the above-referenced patent application:

*Please replace the indicated paragraphs with the provided corrected paragraph:*Page 6, line 19, through page 7, line 5

Figures 17A and 17B are SELDI analyses of microdissected esophageal epithelium showing proteins dysregulated in a disease-specific manner. Figures 18A and 18B are SELDI analyses of 8 different esophageal cancer cases, where three separate microdissections of eight different patients' matched tumor and normal cells were subjected to SELDI analysis via the use of a hydrophobic interaction C18 binding surface. Each replicate was run in triplicate, giving a total of 72 data points for each protein peak analyzed. The analysis of the protein fingerprint in the low mass region is shown in Figure 17A, the higher mass region in Figure 17B. A representative mass map from one case (case #1) is shown on the left side of each panel with the normal and tumor fingerprint shown (top and bottom, respectively) for each mass region. A gel-like representation is displayed for that particular case as well as the fingerprint for two other cases. Proteins 1, 2, 6, and 7 are labeled for orientation. All cases analyzed in the study set were then subjected to analysis as a ratio of relative intensity of the selected proteins to one another

and the statistical results shown on the right side of each figure. Figure 17A: average C.V. = 12.7%, 29.4% for the normal and tumor microdissections respectively. Figure 17B: average C.V. = 10.5% and 18.9% for the normal and tumor microdissections, respectively.

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Figure 18 shows the SELDI analysis of prostate carcinogenesis. Figure 18A shows a mass map that represents the profile from 1500 normal, pre-invasive neoplasia (PIN) and invasive carcinoma cells acquired by LCM from one case (case #2). Additionally, the corresponding patient-matched stromal cells (1500 cells) were also microdissected for analysis. Figure 18B shows a gel-like image of the raw mass data shown in Panel A. All samples from this patient were run in triplicate, with the representation of one experiment shown. Two proteins, A and B, having molecular weights of 28,000 and 32,000 respectively, were found to be reproducibly differentially expressed in this patient and are indicated in both Figures 18A and 18B. Figure 18C shows the ratio analysis of A vs. B from an additional study set of two other (cases #1 and #3) patient matched tumor and normal prostatic epithelium (average C.V. = 17.2 % and 10.1% for the tumor and normal microdissections, respectively).

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One type of assay that can be performed is a soluble immunoassay, where an antibody specific for a protein of interest is used. The antibody can be labeled with a variety of markers, such as chemiluminescence, fluorescence and radioactive markers. For best results, the assay used should be of high sensitivity, such as a microparticle enzyme immunoassay (MEIA). By applying a calibration curve used to estimate immunodetected molecules in serum, the number of molecules per cell can be estimated. Thus, the presently described methods provide a quantitative immunoassay, which can measure the actual number of the protein molecules of interest *in vivo*.

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The O.C.T embedded tissue blocks were cut into 8 μ m sections with a cryostat. After cutting, the sections were immediately placed on dry ice and then stored at -80°C. Only one section was thawed and dissected at a time, to minimize degradation of proteins. After fixation

in 70% ethanol for 10 s, the section was stained with hematoxylin and eosin, and dehydrated in xylene.

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A 5 The detection limit of the assay for microdissected cellular proteins was 0.004 ng/ml PSA as defined as the concentration two standard deviations above the signal response of a sample free of PSA or a PSA negative tissue. The sensitivity achieved with these criteria was one 30 μ m laser shot (5-7 cells).

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A 6 Gel pieces were excised and washed by end-over-end mixing in 12 ml 30% methanol for 30 minutes (room temperature), washed twice for 30 minutes with 150 μ l of 1:1 acetonitrile/100 mM ammonium bicarbonate pH 8. Each spot was sliced into fourths and rehydrated 10 μ l of 100 mM ammonium bicarbonate pH 8 containing trypsin (2 pmol/ μ l) (Promega modified trypsin). Additional 10 μ l of digestion buffer without trypsin was then added. After incubation at 37°C for 20 hours, the condensate was collected by spinning the tubes briefly and excess liquid was removed into a new tube. Peptides remaining in the gel matrix were extracted twice with 150 μ l 60% aqueous acetonitrile, 0.1% TFA at 30°C for 30 min. The extracted volume was reduced to about 5 μ l using an Eppendorf speed-vac concentrator.

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A 7 Mass spectrometric data regarding tryptic peptides from in-gel digestions were obtained from collision induced dissociation (CID) spectra with a Finnigan-MAT LCQ ion trap mass spectrometer after introduction via a polyamide coated fused silica microcapillary HPLC.

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A 8 Tissue from prostate cancer patients undergoing radical prostatectomy at the Clinical Center of the National Cancer Institute (Bethesda, MD) and at the Mayo Clinic (Rochester, MN) was used. Tissue from the peripheral zone of the prostate was procured, immediately snap frozen, and stored at -80° C. Matching normal and tumor cell lines were prepared from the prostatectomy specimens obtained at the NCI and immortalized as described by Bright et al.,

Cancer Res. 57: 995-1002 (1997). LnCaP and PC3 cells were purchased from the American Type Culture Collection (Manassas, VA). Microdissection was performed and the cells were prepared for 2D-PAGE analysis as described above in Example 2. 2D-PAGE gels were done using alpha-tubulin to normalize the sample load, as described in Example 2. The anti-alpha tubulin antibodies, used at 1:1000, and HRP-coupled rabbit anti-mouse secondary antibodies, used at 1:10,000, were purchased from Sigma (St. Louis, MO). Blots were washed using conditions described above and ECL substrate (Amersham, Piscataway, NJ) was added for chemiluminescent detection via autoradiography on Kodak Bio-Max film. For PSA, anti-PSA antibodies were purchased from Scripps Laboratories, San Diego, CA.

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A⁹ To determine if the protein profiles from prostate cancer cell lines are representative of prostate cancer in vivo, lysates of cells cultured in vitro were subjected to 2D-PAGE and the resulting patterns compared to those of LCM-derived normal malignant prostatic epithelium. The 2D-PAGE pattern of two common cell lines, LnCaP and PC3, were first compared to the pattern of in vivo cancerous cells. Protein expression between the two cell lines was similar, but markedly different from the protein profiles of epithelium in vivo, exhibiting less than 20% identity. To assess whether differences in 2D-PAGE profiles were the result of qualitative or quantitative alterations in expression, immunoblots were done using PSA as a test molecule. PSA was not detected in normal cells, PC3, or a tumor cell line developed from the in vivo dissected tumor cells. In contrast, LnCaP expressed PSA, but there was an alteration in the migration, reflective of a qualitative change in the protein. These results indicate that there are both quantitative and qualitative changes in cell expression when comparing immortal and in vivo isolated cells. Finally, a direct comparison between the in vivo tumor cells pre-immortalization and a cell line developed from those tumor cells shows substantial alterations in protein expression. This observation means at least some of the differences in protein expression seen between the LnCaP and PC3 cell lines and the tumor cells reflect of changes in protein expression due to the immortalization process.

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A¹⁰
Frozen tissue was obtained from radical prostatectomy specimens and embedded in OCT compound (Tissue-Tek, Miles, Elkhart, IN). Eight micron sections were made with a standard cryostat and stained with hematoxylin and eosin using standard protocols. Benign and malignant histology was identified by a pathologist and LCM was performed to obtain cells from each population by directing the laser at those populations of cells. LCM was performed as previously described, except AEBSF (Boeringer Mannheim) was added to the staining baths at a final concentration of 2 mM to inhibit proteases. For 1-dimensional and 2-dimensional electrophoresis analysis 2,000 (approximately 8 - 10,000 cells) and 5,000 (approximately 20 - 25,000 cells) 30 micron laser shots of each cell population were used, respectively. Based on careful review of histologic sections each dissection is estimated to contain > 95% of desired cells.

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A¹¹
PSA is a serine protease that is produced as an inactive zymogen and then activated by release of a signal peptide of 17 amino acids followed by liberation of a 7 amino acid propeptide. The catalytically active form of PSA is highly glycosylated with a molecular mass of approximately 30 kd. Although it has been shown that PSA is constitutively produced in LnCaP cells, the relative proportion of pre and processed forms of PSA within uncultured prostatic epithelium has not been reported. Furthermore, the site at which PSA binds to ACT is also not known. In order to answer these questions, anti-PSA western blot analysis was performed on cellular lysates from LCM derived normal and malignant epithelial cells, which revealed a band at 30kd (Figure 10). These results were highly reproducible in several cases and demonstrate that a majority of intracellular PSA exists as a 30 kd form. This finding suggests that PSA binds to ACT within either the extracellular space or the serum and not intracellularly. The total abundance of cellular PSA varied such that in some cases the malignant cells contained more PSA while in other cases the benign cells contained more PSA. This variability of intracellular PSA levels within both normal and malignant prostatic epithelium is consistent with previous reports.

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A¹² To ascertain whether the 30 kd protein represents the active form of PSA we performed experiments to determine if it would bind to ACT. As shown in Figure 11, the majority of cellular PSA complexed with ACT after 2 hours of incubation and there was no difference in the binding capacity of PSA derived from normal and malignant epithelium. This finding suggests that the 30 kd form represents active PSA but does not explain why serum percent free PSA is lower in men with cancer. Although the PSA found intracellularly in normal and malignant epithelial cells appeared identical by molecular weight and ACT binding capacity, other protein modifications that do not alter patterns on 1D PAGE gels may have been missed by this investigative methodology. Therefore, 2D PAGE was performed on cellular lysates from LCM derived tissue. As can be seen in Figure 12A, three distinct 30 kd PSA isoforms similarly exist in benign and malignant epithelial cells indicating that the PSA found in malignant prostatic epithelium is not mutated or differentially glycosylated, because this would be reflected in a change in isoelectric focusing point on a 2D Western. Furthermore, none of these three isoforms are a proteolytically cleaved form of PSA since all remained intact under reducing conditions. The fact that the purified PSA/ACT complex remains intact under the reducing conditions (Figure 12B) validates that the findings demonstrated in Figure 11 are not an artifact of 2D - PAGE. In addition, whole tissue frozen sections were analyzed (that had not been exposed to LCM) containing both benign and malignant epithelium to demonstrate that firing the laser on the cells did not alter PSA structure (Figure 12A).

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A¹³ To assess the reproducibility of the protein fingerprint generated by SELDI, two separate cell populations of prostatic tumor epithelial cells (1200 cells per dissection) from the same patient (Case 1) were separately microdissected (Microdissections 1 and 2) from frozen tissue sections. AEBSF (Boehringer Mannheim) was added to the staining baths at a final concentration of 2 mM to inhibit proteases. Based on careful review of the histologic sections by a pathologist, each microdissection was estimated to have greater than 95% purity. Microdissected cells were lysed directly on an LCM cap with 2 μ l of an extraction buffer containing 1% (w/v) Triton-X-100 (Sigma, St. Louis, MO), 1% (w/v) MEGA 10 (ICN, Aurora, OH), and 1% (w/v) octyl-B-glucopyranoside (ESA, Chelmsford, MA) in a standard 1X PBS

base. SELDI analysis was performed using an aliphatic reverse phase chip (Ciphergen, Palo Alto, CA.). The bait surfaces on the chip were pretreated with 2 μ l of acetonitrile (Sigma, St. Louis, MO). Shortly before the acetonitrile completely evaporated, 2 μ l of the lysate was applied to the bait surface. The analyte was allowed to concentrate by air drying followed by the application of 0.3 ml of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, 98%, Sigma, St. Louis, MO) as the energy absorbing molecule of choice for all experiments in this study.

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A¹⁴ For the purposes of these studies only the molecular weight range of 15 kDa to 70 kDa was analyzed, although the SELDI has a practical resolution and detection working range of 1000 to 300,000 daltons depending on the energy absorbing molecule utilized and the bait surface/wash conditions employed. Western blot analysis of LCM derived cells in our laboratory using anti alpha-tubulin as a housekeeping marker for a protein expression reference standard indicated that procurement of cells by an equal number of laser shots generates a nearly equivalent (+/- 5%) final protein yield (data not shown).

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A¹⁵ To apply the present methods to this type of analysis, the tissue from multiple patients is exposed to the agent to be tested. The exposure can be done *in vivo*, prior to collection of the sample, or *in vitro*, after collection of the sample and/or after laser microdissection. *In vivo* exposure would involve administration of the agent to the subject, while *in vitro* administration could be administration to cultured cells. Some agents that could be tested include pharmacological agents, imaging agents, labeled proteins, such as ligands, or other agents known to have particular effects on cells, such as cytokines. After exposure, microdissection techniques are used to isolate the cells of interest from the sample, the cells are lysed to allow isolation of the proteins or other cellular components, such as nucleic acids or other subcellular structures, from the sample, and the lysate contents are transferred to a confined zone of a substrate. The lysate contents, or cellular components, are placed in identifiable positions on a substrate, where such positions are confined zones. One example of a confined zone is the coordinates of an array. The array is constructed by applying microspots of the isolated proteins on any suitable matrix, such as nitrocellulose, nylon, or silica.

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A 16 Referring to Figure 19, the microspots are arranged on the matrix in any manner that produces meaningful data. The microspots can be placed on the matrix using, for example, a micropipette, and examples of the substrate include a glass or plastic slide, a section of embedding medium, or a nitrocellulose matrix. In one embodiment, the microspots can be arranged in the y-dimension by patient number and in the x-dimension by a criterion for categorizing the various samples obtained, such as by stage of malignancy. Other alternative criteria include before and after treatment samples, various cells types, such as epithelial and stromal, or stages of development for embryonic samples. The microspots are then subjected to some type of analysis, for example, to determine if an amount of a particular protein is altered. Such analysis can include probing with an antibody as the binding agent. Alternative analyses include probing with other binding agents such as nucleic acids, labeled or unlabeled DNA or RNA, or aptameric or phage display screening. A consistent alteration in the cellular content of a protein is then correlated to exposure of the tissue or cells to the agent of interest.